

BH3-Only Protein Bid Participates in the Bcl-2 Network in Healthy Liver Cells

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Bcl-2 homology domain 3 (BH3)-only protein Bid is posttranslationally cleaved by caspase-8 into its truncated form (tBid) and couples with stress signals to the mitochondrial cell death pathway. However, the physiological relevance of Bid is not clearly understood. Hepatocyte-specific knockout (KO) of Bcl-xL leads to naturally-occurring apoptosis despite co-expression of Mcl-1, which shares a similar anti-apoptotic function. We generated Bcl-xL KO, Bcl-xL/Bid double KO, Bcl-xL/Bak double KO, Bcl-xL/Bax double KO, and Bcl-xL/Bak/Bax triple KO mice and found that hepatocyte apoptosis caused by Bcl-xL deficiency was completely dependent on Bak and Bax, and surprisingly on Bid. This indicated that, in the absence of Bid, Bcl-xL is not required for the integrity of differentiated hepatocytes, suggesting a complicated interaction between core Bcl-2 family proteins and BH3-only proteins even in a physiological setting. Indeed, a small but significant level of tBid was present in wild-type liver under physiological conditions. tBid was capable of binding to Bcl-xL and displacing Bak and Bax from Bcl-xL, leading to release of cytochrome c from wild-type mitochondria. Bcl-xL-deficient mitochondria were more susceptible to tBid-induced cytochrome c release. Finally, administration of ABT-737, a pharmacological inhibitor of Bcl-2/Bcl-xL, caused Bak/Bax-dependent liver injury, but this was clearly ameliorated with a Bid KO background. **Conclusion:** Bid, originally considered to be a sensor for apoptotic stimuli, is constitutively active in healthy liver cells and is involved in the Bak/Bax-dependent mitochondrial cell death pathway. Healthy liver cells are addicted to a single Bcl-2-like molecule because of BH3 stresses, and therefore special caution may be required for the use of the Bcl-2 inhibitor for cancer therapy. (HEPATOLOGY 2009;50:1972-1980.)

Abbreviations: ALT, alanine aminotransferase; BH3, Bcl-2 homology domain 3; KO, knockout; tBid, truncated form of Bid; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling.

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Bcl-2 family proteins regulate the mitochondrial pathway of apoptosis in mammalian cells.¹ They are divided into two basic groups: core Bcl-2 family proteins and Bcl-2 homology domain 3 (BH3)-only proteins. Core Bcl-2 family proteins have three or four Bcl-2 homology domains (BH1-BH4 domains), referred to as multidomain members, and structural similarity. These proteins display opposing bioactivities from inhibition to promotion of apoptosis and can be further divided into two groups: anti-apoptotic members, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and Bfl-1, and pro-apoptotic members, including Bax and Bak. Pro-apoptotic Bak and Bax are effector molecules of the Bcl-2 family and induce release of cytochrome c from mitochondria, presumably through their ability to form pores at the mitochondrial outer membrane. Anti-apoptotic members, which serve as regulators, inhibit Bak and Bax. The original rheostat model argues for a fine balance between Bax-like pro-apoptotic proteins and Bcl-2-like an-

ti-apoptotic proteins in defining life and death, and this balance would be equal or favor survival in a healthy cell.²

BH3-only proteins consist of at least eight members and only share homology with each other and the core Bcl-2 family proteins through the short BH3 motif. They are transcriptionally induced or posttranslationally activated in response to a variety of apoptotic stimuli.³ When they are induced or activated, they interact with core Bcl-2 family proteins and set the rheostat balance toward apoptosis by directly activating Bax-like molecules or neutralizing Bcl-2-like molecules.⁴ Therefore, they serve as initial sensors of apoptotic signals that emanate from various cellular processes. Bid, a member of the BH3-only proteins, is activated via caspase-8-mediated cleavage in response to ligation of the death receptor, and its N-terminal truncated form (tBid) translocates to mitochondria and activates the mitochondrial death pathway.⁵ In so-called type 1 cells, such as lymphoid cells, Fas activation leads to caspase-8 activation followed by direct activation of downstream caspases such as caspase-3 and caspase-7, where Bid does not have significant roles.⁶ In contrast, in type 2 cells, Fas-mediated activation of caspase-8 is not enough to activate downstream caspases. In those cells, tBid links the extrinsic or death-receptor pathway to the intrinsic or mitochondrial pathway to execute apoptosis. Hepatocytes are identified as a typical type 2 cell in which Bid plays a critical role in receptor-mediated cell death pathways.⁷

In our previous research, we found that genetic ablation of Bcl-xL in hepatocytes causes spontaneous apoptosis in mice.⁸ This indicates that Bcl-xL is a critical apoptosis antagonist in adult healthy hepatocytes, although they possess other anti-apoptotic members of the Bcl-2 family such as Mcl-1. This might be simply explained by the fact that the absence of Bcl-xL affects the rheostat balance of core Bcl-2 family proteins by increasing the ratio of Bax and Bak to anti-apoptotic Bcl-2 proteins. Indeed, neuronal cell death during development caused by Bcl-xL deficiency is ameliorated by loss of Bax.⁹ Platelet cell death caused by Bcl-xL deficiency is also ameliorated by loss of Bak.¹⁰ These studies indicate that the stoichiometry between Bcl-xL and Bax or Bak dictates cellular fate. However, the possibility of BH3-only proteins being involved in the apoptosis rheostat in healthy cells has not been addressed. We generated Bcl-xL/Bid double-knockout (KO) mice and demonstrated that apoptosis caused by Bcl-xL deficiency is critically dependent on Bid. A small amount of Bid appears to be activated in the liver under physiological conditions and to be significant for inducing cytochrome c release from Bcl-xL-deficient mitochondria. This study shed light on the active participation of BH3-only proteins, which are generally

considered to be sensors of apoptotic stimuli, in the Bcl-2 network regulating life and death of healthy differentiated hepatocytes.

Materials and Methods

Mice. Mice carrying a *bcl-x* gene with 2 loxP sequences at the promoter region and a second intron (*bcl-x^{lox/lox}*) were described previously.¹¹ Heterozygous AlbCre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene⁸ and traditional Bid KO mice⁷ also have been described previously. We purchased from the Jackson Laboratory (Bar Harbor, ME) traditional Bak KO mice, traditional Bax KO mice, and conditional Bak/Bax KO mice (*bak^{-/-} bax^{lox/lox}*).¹² We generated hepatocyte-specific Bcl-xL KO mice (*bcl-x^{lox/lox} AlbCre*), Bcl-xL/Bid double-KO mice (*bid^{-/-} bcl-x^{lox/lox} AlbCre*), Bcl-xL/Bak double-KO mice (*bak^{-/-} bcl-x^{lox/lox} AlbCre*), Bcl-xL/Bax double-KO mice (*bax^{-/-} bcl-x^{lox/lox} AlbCre*), and Bcl-xL/Bak/Bax triple-KO mice (*bak^{-/-} bax^{lox/lox} bcl-x^{lox/lox} AlbCre*) by mating the strains. They were maintained in a specific pathogen-free facility and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. The levels of serum alanine aminotransferase (ALT) were measured by a standard method, and serum caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Tokyo, Japan). The caspase-3/7 activity was normalized by each control group. For histological analysis, the liver sections were stained with hematoxylin-eosin. To detect cells with oligonucleosomal DNA breaks, the sections were also subjected to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining, according to a previously reported procedure.¹³

Western Blot Analysis. Liver tissue was lysed with a lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 × protein inhibitor cocktail (Nacalai tesque, Kyoto, Japan), phosphate-buffered saline, pH 7.4). Equal amounts of protein were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride membrane. For immunodetection, the following antibodies were used: anti-Bcl-xL antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 antibody (Rockland, Gilbertsville, PA), previously described anti-Bid antibody generated from glutathion-S-transferase-Bid fusion protein,¹⁴ anti-full-length Bid antibody, anti-cleaved caspase-7 antibody, anti-Bax antibody, anti-Cox IV antibody (Cell Signaling Technology, Beverly, MA),

anti-Bak antibody (Millipore, Billerica, MA), and anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO).

Isolation of Mitochondria-Rich and Cytosolic Fraction. After liver tissue was homogenized using isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid, 1 mg/mL fatty acid-free bovine serum albumin, 1 \times protein inhibitor cocktail, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid-potassium hydroxide, pH 7.4), the lysate was centrifuged at 600g for 10 minutes, and the supernatant was centrifuged at 15,000g for 10 minutes. The pellet was regarded as a mitochondria-rich fraction and the supernatant as a cytosolic fraction.

Immunoprecipitation of Bcl-xL. Approximately 30 mg liver tissue was lysed with a TNE buffer (1% Nonidet P-40, 1 mM ethylenediaminetetra-acetic acid, 1 \times protein inhibitor cocktail, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.8). Equal amounts of protein samples were rotated with protein G sepharose (GE Healthcare, Tokyo, Japan) and anti-Bcl-xL antibody (Abcam, Cambridge, MA) overnight at 4°C. After centrifugation, the pellet was collected as the immunoprecipitate protein.

Incubation of tBid or Bid for Immunoprecipitation. Liver tissue (90 mg) was lysed with 800 μ L lysis buffer (2 mM ethylenediaminetetra-acetic acid, 10 mM ethylene glycol tetra-acetic acid, 50 mM NaF, 5 mM Na₂P₄O₇, 10 mM β -glycerophosphate, 0.1% 2-mercaptoethanol, 1% Triton X, 1 \times protein inhibitor cocktail, 50 mM Tris-HCl, pH 7.5). Equal volumes of protein samples were incubated with or without recombinant mouse tBid or full-length Bid (R&D Systems, Minneapolis, MN).

Analysis of Cytochrome C Release. The mitochondria-rich fraction was diluted in a mitochondria dilution buffer (395 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid-potassium hydroxide, pH 7.4). The diluted mitochondria were incubated with recombinant mouse tBid or full-length Bid diluted with a reaction buffer (125 mM KCl, 0.5 mM MgCl₂, 3.0 mM succinic acid, 3.0 mM glutamic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid-potassium hydroxide, 1 \times protein inhibitor cocktail, 2.5 mM ethylenediaminetetra-acetic acid and BOC-Asp (OMe) CH₂F 20 μ M, pH 7.4) for 30 minutes at 37°C. The levels of cytochrome c in the buffer were determined using an enzyme-linked immunosorbent assay kit (R&D Systems). The maximum or spontaneous release of cytochrome c was defined as the level of samples incubated with 0.1% Triton X-100 or medium alone, respectively. The percentage release of cytochrome c was calculated using the following formula:

% release = (experimental release - spontaneous release) \times 100/(maximum release - spontaneous release).

ABT-737 Injection Study. ABT-737 was provided

by Abbott Laboratories (Abbott, Park, IL). ABT-737 was dissolved with a mixture of 30% propylene glycol, 5% Tween 80, and 65% D5W (5% dextrose in water), final pH 4 to 5. Mice were given a single intraperitoneal injection of ABT-737 at 100 mg/kg and sacrificed 16 hours later. Platelets were counted using an automated cell counter (Sysmex, Kobe, Japan).

Statistical Analysis. Data are presented as mean \pm standard deviation. Multiple comparisons of TUNEL-positive cells were performed by analysis of variance followed by Fisher's *post hoc* correction. The other multiple comparisons were performed by analysis of variance followed by Scheffe *post hoc* correction. $P < 0.05$ was considered statistically significant.

Results

Hepatocyte Apoptosis Caused by Bcl-xL Deficiency Is Completely Lost with Bid-Deficient Background.

To examine the possibility of whether Bid is involved in apoptosis caused by Bcl-xL deficiency, hepatocyte-specific Bcl-xL KO mice were crossed with traditional Bid KO mice. After mating *bid*^{+/-} *bcl-x*^{flx/flx} *AlbCre* mice with *bid*^{+/-} *bcl-x*^{flx/flx} mice, western blot analysis confirmed lack of Bcl-xL and Bid in the liver of Bcl-xL KO mice and Bid KO mice, respectively, and intermediate expression of Bid in the Bid +/- liver (Fig. 1A). Consistent with our previous findings,⁸ Bcl-xL KO mice (*bid*^{+/+} *bcl-x*^{flx/flx} *AlbCre*) produced spontaneous hepatocyte apoptosis (Fig. 1B), which was associated with caspase-7 activation in the liver (Fig. 1C). Serum ALT levels (Fig. 1D), caspase-3/7 activity (Fig. 1E), and the frequency of TUNEL-positive hepatocytes (Fig. 1F) were significantly higher in Bcl-xL KO mice than in wild-type mice (*bid*^{+/+} *bcl-x*^{flx/flx}). Bid KO mice (*bid*^{-/-} *bcl-x*^{flx/flx}) did not produce any liver phenotypes under physiological conditions, in agreement with a previous report.⁷ This was further confirmed by our additional analysis on Bid KO mice and control littermates, which showed no difference in serum ALT levels (Supporting Fig. 1A), caspase-3/7 activity (Supporting Fig. 1B), and the ratios of liver weight to body weight (Supporting Fig. 1C). Of importance is the finding that serum ALT levels were reduced to the normal levels in Bcl-xL/Bid double-KO mice (*bid*^{-/-} *bcl-x*^{flx/flx} *AlbCre*). Bcl-xL KO with Bid heterozygosity (*bid*^{+/-} *bcl-x*^{flx/flx} *AlbCre*) displayed intermediate ALT levels between Bcl-xL KO mice and double-KO mice. In agreement with this observation, the number of TUNEL-positive hepatocytes in Bcl-xL/Bid double-KO mice reached background levels. In addition, the levels of caspase-3/7 activity in serum were also normalized in Bcl-xL/Bid double-KO mice. Taken together, these observations indicated that

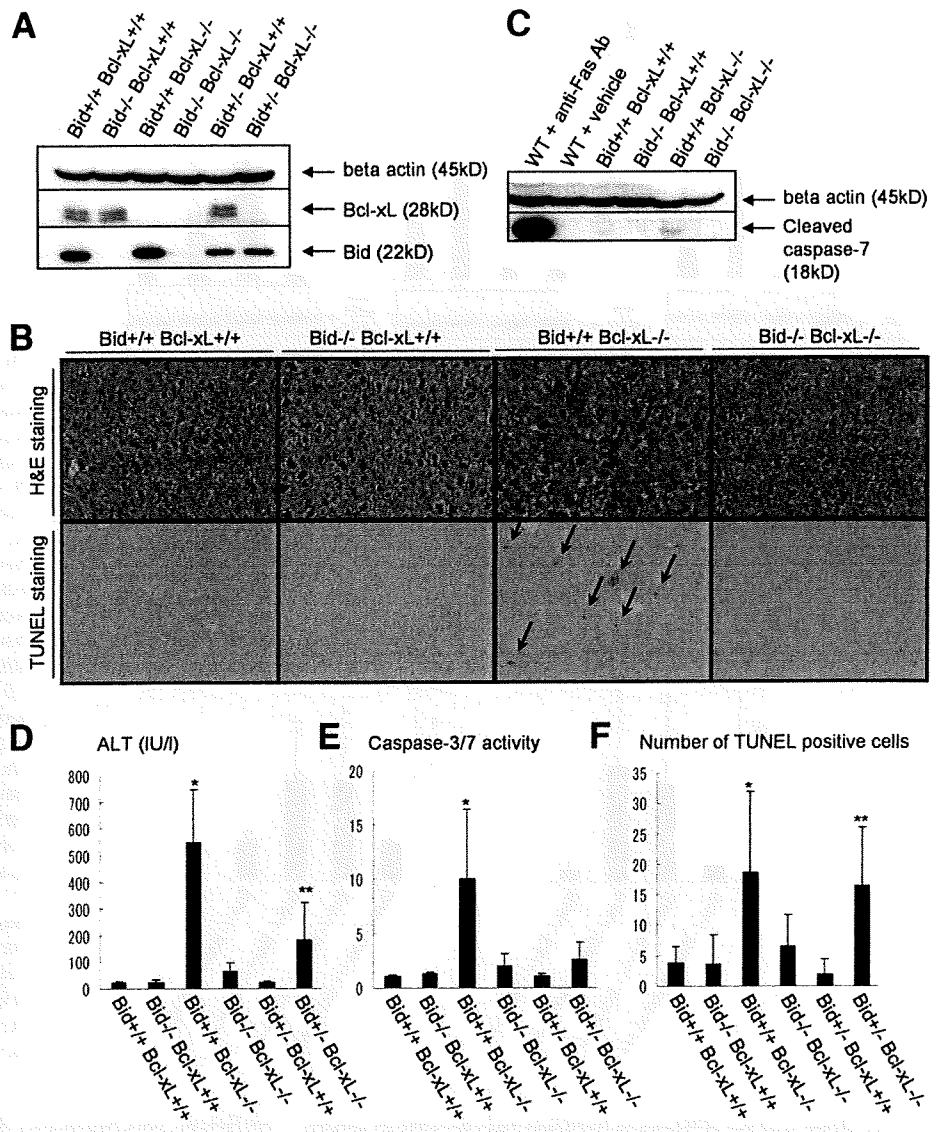


Fig. 1. Bcl-xL/Bid double-KO mice. Offspring from mating *bid*^{+/-} *bcl-x*^{fllox/fllox} *AlbCre* mice and *bid*^{+/-} *bcl-x*^{fllox/fllox} mice were sacrificed at 6 weeks after birth. Bcl-xL^{+/+} stands for *bcl-x*^{fllox/fllox} without *AlbCre*, and Bcl-xL^{-/-} stands for *bcl-x*^{fllox/fllox} with *AlbCre*. (A) Western blot of whole liver lysate for the expression of Bcl-xL and Bid. (B) Representative pictures of liver histology stained with hematoxylin-eosin and TUNEL. Arrows indicate typical apoptotic cells. (C) Western blot of whole liver lysate for the expression of cleaved caspase-7. Live lysates from wild-type mice 3 hours after intraperitoneal injection of 30 μg anti-Fas antibody (clone Jo2) or vehicle were included as a positive and a negative control, respectively. (D) Serum ALT levels. N = 14 mice per group. **P* < 0.05 versus the other five groups; ***P* < 0.05 versus the other four groups except Bid^{-/-} Bcl-xL^{-/-} group. (E) Serum caspase-3/7 activity. N = 13 mice per group. **P* < 0.05 versus the other five groups. (F) Statistics of TUNEL-positive cells. N = 6 mice per group. * and ***P* < 0.05 versus all of Bcl-xL^{+/+} groups and Bid^{-/-} Bcl-xL^{-/-} group.

apoptosis caused by Bcl-xL deficiency is completely dependent on the BH3-only protein Bid. Bid is activated by tumor necrosis factor (TNF) receptor,¹⁵ and TNF-α, which is a ligand of TNF receptor, is produced by Myd88 signal pathway.¹⁶ To examine the possibility of involvement of Myd88 or TNF-α in this apoptosis, we generated Myd88 Bcl-xL double-KO mice by crossing *myd88*^{-/-} mice with *bcl-x*^{fllox/fllox} *AlbCre* mice and administered neutralizing anti-TNF-α antibody into Bcl-xL KO mice. Hepatocyte apoptosis caused by Bcl-xL deficiency was not ameliorated with Myd88 KO background or by administration of anti-TNF-α antibody (Supporting Fig. 2A, B).

Hepatocyte Apoptosis Caused by Bcl-xL Deficiency Requires Both Bak and Bax. To depict the precise relationships among core Bcl-2 family proteins in regulating liver homeostasis, hepatocyte-specific Bcl-xL-deficient mice were crossed with traditional Bak or Bax KO

mice. The levels of serum ALT were slightly decreased with a Bak KO background (*bak*^{-/-} *bcl-x*^{fllox/fllox} *AlbCre*), whereas they did not change with a Bax KO background (*bax*^{-/-} *bcl-x*^{fllox/fllox} *AlbCre*) (Fig. 2A, B). To examine the contribution of both Bax and Bak, Bcl-xL KO mice were crossed with conditional Bak/Bax KO mice. The levels of serum ALT were completely normalized in Bak/Bax KO background (*bak*^{-/-} *bax*^{fllox/fllox} *bcl-x*^{fllox/fllox} *AlbCre*) (Fig. 2C). Hepatocyte apoptosis determined by TUNEL staining of liver sections and caspase activation determined by caspase-3/7 activity in serum also returned to background levels (Fig. 2D, E). These observations clearly indicated that apoptosis caused by Bcl-xL deficiency was generated through the Bak/Bax-dependent mitochondrial cell death pathway. To clarify the background levels of hepatocyte apoptosis, we also analyzed the liver apoptosis in *bak*^{-/-} and *bak*^{-/-} *bax*^{fllox/fllox} *AlbCre* mice. Similarly, in *bid*^{-/-}

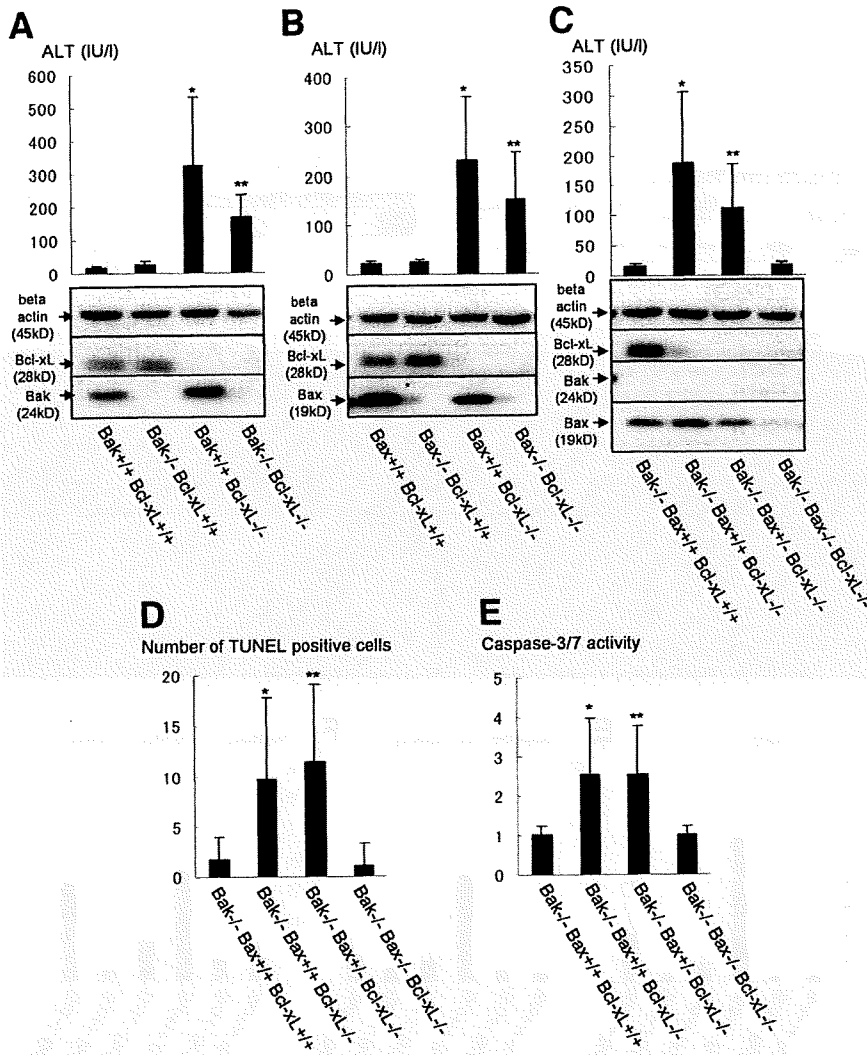


Fig. 2. Bcl-xL KO mice with Bak or Bax KO background. (A) Offspring from mating *bak*^{+/+} *bcl-x*^{fllox/fllox} AlbCre mice and *bak*^{+/+} *bcl-x*^{fllox/fllox} mice were sacrificed at 6 weeks after birth. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL and Bak are shown. N = 14 mice per group. * and ***P* < 0.05 versus the other three groups. (B) Offspring from mating *bax*^{+/+} *bcl-x*^{fllox/fllox} AlbCre mice and *bax*^{+/+} *bcl-x*^{fllox/fllox} mice were sacrificed at 6 weeks after birth. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL and Bax are shown. N = 15 mice per group. * and ***P* < 0.05 versus the other two Bcl-xL+/+ groups. (C, D, and E) Offspring from mating *bak*^{-/-} *bax*^{fllox/+} *bcl-x*^{fllox/fllox} AlbCre mice and *bak*^{-/-} *bax*^{fllox/+} *bcl-x*^{fllox/fllox} mice were sacrificed at 6 weeks after birth. Bax+/+ stands for -*bax*^{fllox/fllox} without AlbCre, and Bax-/- stands for *bax*^{fllox/fllox} with AlbCre. N = 8 or 10 mice per group. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL, Bak, and Bax are shown (C). **P* < 0.05 versus Bak-/- Bax+/+ Bcl-xL+/+ and Bak-/- Bax-/- Bcl-xL-/- groups; ***P* < 0.05 versus Bak-/- Bax-/- Bcl-xL-/- group. Statistics of TUNEL-positive cells (D). * and ***P* < 0.05 versus Bak-/- Bax+/+ Bcl-xL+/+ and Bak-/- Bax-/- Bcl-xL-/- groups. Serum caspase-3/7 activity (E). * and ***P* < 0.05 versus Bak-/- Bax+/+ Bcl-xL+/+ and Bak-/- Bax-/- Bcl-xL-/- groups.

mice, there was no difference between two groups in serum ALT levels (Supporting Fig. 3A), caspase-3/7 activity (Supporting Fig. 3B), and the ratios of liver weight to body weight (Supporting Fig. 3C), which suggests that healthy hepatocytes in wild-type mice are completely protected from Bid or Bak/Bax-mediated apoptosis by Bcl-xL.

Bcl-xL Interacts with Cytosolic Bax and Mitochondrial Bak in the Liver. To examine the expression of a variety of Bcl-2-related molecules in the liver, cytosolic and mitochondrial fractions from liver lysate were subjected to western blot analysis (Fig. 3A). Anti-apoptotic Bcl-2 proteins, Bcl-xL and Mcl-1, were expressed at both the mitochondria and the cytosol. In contrast, Bak and Bax were exclusively expressed at the mitochondria and the cytosol, respectively. Full-length Bid was expressed mainly in the cytosol. To examine whether Bcl-xL physically interacts with those Bcl-2-related proteins, liver lysate was immunoprecipitated with Bcl-xL and identified using corresponding antibodies (Fig. 3B). At least a part

of Bcl-xL was bound to Bak and Bax, but not to Mcl-1 or full-length Bid.

tBid, But Not Full-Length Bid, Displaces Bak and Bax from Bcl-xL by Binding to Bcl-xL. Bcl-2-like molecules have been shown to be capable of binding Bak or Bax, and through this interaction, to neutralize each activity.¹⁷ Conversely, other research showed that Bcl-xL does not have to bind Bax-like molecules to protect against cell death.¹⁸ To examine the impact of tBid on the association between Bcl-xL and Bak or Bax, we added tBid to the liver lysate and examined the interaction of each Bcl-2-related protein with Bcl-xL by immunoprecipitation. Addition of 500 nM tBid abolished the association between Bcl-xL and Bak or Bax (Supporting Fig. 4). Simultaneously, Bcl-xL binding of tBid was observed. Addition of 20 nM tBid also abolished, if not completely, the association between Bcl-xL and Bak or Bax (Fig. 4). In contrast, adding the same concentration of full-length Bid had little effect on Bcl-xL binding of Bak or Bax (Fig. 4). These results indicated that tBid can bind to Bcl-xL

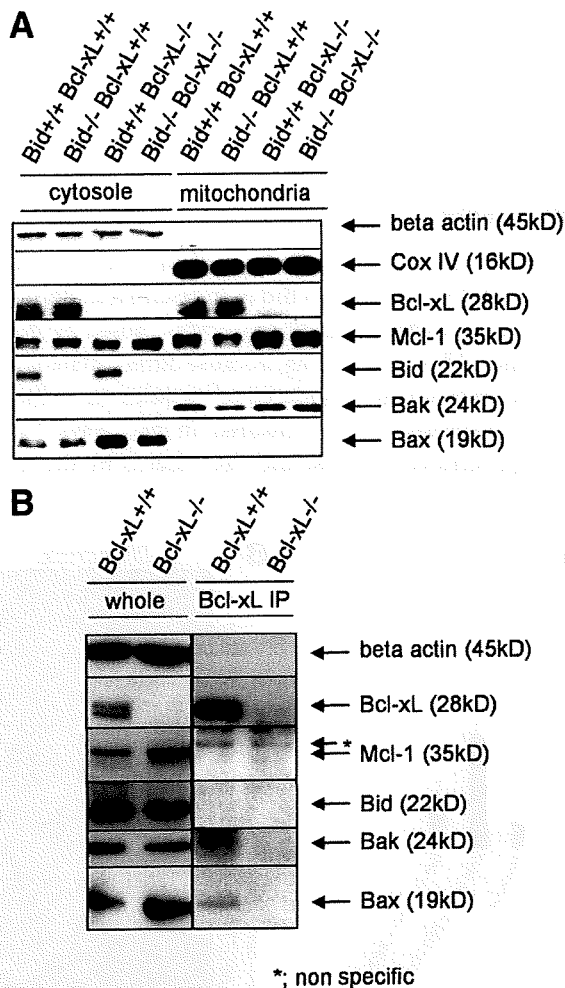


Fig. 3. Expression of Bcl-2-related molecules in the liver and their association with Bcl-xL. Bcl-xL^{+/+} stands for *bcl-x^{fllox/fllox}* without *AlbCre*, and Bcl-xL^{-/-} stands for *bcl-x^{fllox/fllox}* with *AlbCre*. (A) Western blot after cellular fractionations of the liver lysate. Loading amounts of cytosolic and mitochondrial fractions were adjusted to be equivalent for the starting liver samples. (B) Western blot after anti-Bcl-xL immunoprecipitation. Whole cellular lysate and immunoprecipitates with anti-Bcl-xL were verified with the indicated antibodies. Samples from Bcl-xL^{-/-} mice were included as a negative control.

and suggest that tBid binding of Bcl-xL unleashes Bak or Bax from Bcl-xL.

A Small But Significant Level of tBid Is Detected in the Healthy Liver. Genetic evidence that Bid is required for Bak/Bax-dependent apoptosis caused by Bcl-xL deficiency and biochemical evidence that full-length Bid is inactive for displacing Bak or Bax from Bcl-xL together suggest that tBid is produced in wild-type liver. To confirm this, we performed western blot analysis using antibody that can detect tBid (Fig. 5A). Liver lysate from Bid KO mice served as a negative control, whereas that from wild-type mice injected with anti-Fas antibody served as a positive control. A significant level of tBid was detected in wild-type liver, although

the amount was smaller than in Fas-stimulated mice, which displayed massive live cell apoptosis.

Bcl-xL-Deficient Mitochondria Are Susceptible to a Trace Amount of tBid. To examine the impact of a small amount of tBid on Bcl-xL-deficient mitochondria, tBid or full-length Bid at various concentrations was incubated with mitochondria isolated from Bcl-xL-deficient liver or wild-type liver (Fig. 5B). In agreement with previous reports,¹⁹ wild-type mitochondria efficiently released cytochrome c on exposure to tBid. Full-length Bid was far less effective at releasing cytochrome c. Importantly, Bcl-xL-deficient mitochondria were capable of releasing cytochrome c on exposure to a smaller amount of tBid than wild-type mitochondria. This agrees with the in vivo findings that Bcl-xL-deficient hepatocytes, but not wild-type hepatocytes, underwent apoptosis with a trace amount of tBid.

Administration of ABT-737 Produces ALT Elevation in Wild-Type Mice But to a Lesser Extent in Bid KO Mice. Bcl-2-like molecules have been receiving attention as a target for inducing apoptosis, especially in cancer cells.²⁰ A variety of BH3 mimetics that interact with the hydrophobic groove of anti-apoptotic Bcl-2 proteins has been developed. They inhibit binding of anti-apoptotic Bcl-2-like molecules with BH3-only proteins and presumably with Bak and Bax. ABT-737, a prototype of this class of agents, was designed to mimic the BH3-only protein Bad and can inhibit the function of Bcl-2, Bcl-xL, or Bcl-w but not that of Mcl-1.²¹ Our data on Bcl-xL KO mice raised the possibility that pharmacological inhibition of Bcl-xL may cause hepatocyte apoptosis. To examine this possibility, we injected ABT-737 and examined the liver injury. As expected, the levels of ALT

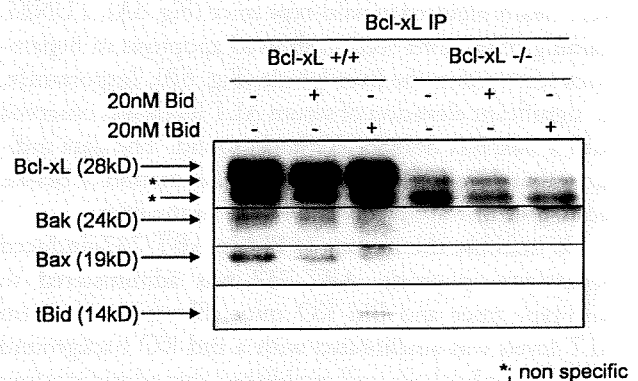


Fig. 4. tBid binds to Bcl-xL and displaces Bak or Bax from Bcl-xL. Liver lysate from *bcl-x^{fllox/fllox}* without *AlbCre* (Bcl-xL^{+/+}) and *bcl-x^{fllox/fllox}* with *AlbCre* (Bcl-xL^{-/-}) were incubated with or without 20 nM recombinant tBid or recombinant full-length Bid at 37°C for 20 minutes. After immunoprecipitation with Bcl-xL, immunoprecipitates are verified with indicated antibodies. Immunoprecipitated lysate from Bcl-xL^{-/-} mice was loaded as a negative control.

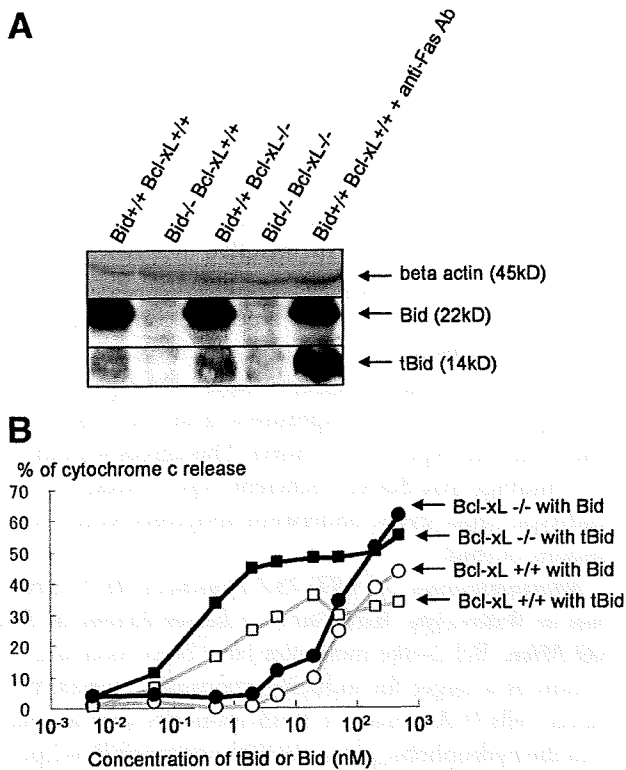


Fig. 5. A small amount of tBid is expressed in wild-type liver and is sufficient for producing cytochrome c release from Bcl-xL-deficient mitochondria. (A) Western blot of liver lysate for Bid and tBid expression. Lysate from wild-type (Bid+/+ Bcl-xL+/+) mice 1 hour after intravenous injection of 10 μ g anti-Fas antibody (clone Jo2) and from Bid-/- mice were included as a positive and a negative control of tBid, respectively. (B) Mitochondrial release of cytochrome c to tBid. Mitochondria were isolated from Bcl-xL-deficient or wild-type liver and incubated with recombinant tBid or recombinant full-length Bid at various concentrations for 30 minutes. Similar results were obtained in three times repeated experiments.

were clearly elevated in wild-type mice (Fig. 6A). TUNEL staining of the liver section showed apoptosis in hepatocytes scattered in the liver lobule (Fig. 6B). Importantly, no significant elevation of serum ALT levels was observed with a Bak/Bax double-KO background. The data indicated that genetic and pharmacological ablation of Bcl-xL led to a similar apoptosis phenotype in the liver.

To examine the impact of Bid in ABT-737-induced hepatocyte apoptosis, ABT-737 was administered to wild-type mice and Bid KO mice. Elevation of serum ALT levels was ameliorated with a Bid KO background (Fig. 6C). It has been well established that administration of ABT-737 led to acute thrombocytopenia.²² This was explained by the fact that Bcl-xL is a critical apoptosis antagonist in platelets.¹⁰ In our experiment, the counts of circulating platelets declined significantly in the wild-type mice (Fig. 6D), which is in the agreement with previous studies.¹⁰ Interestingly, a similar degree of thrombocyto-

penia was observed even in Bid KO mice, suggesting that Bid does not play a significant role in regulating platelet homeostasis, unlike in hepatocytes. The data imply that the impact of Bid in the Bcl-2 network in healthy cells is cell-type specific.

Discussion

One of the important findings of the current study is that the BH3-only protein Bid is an essential molecule for apoptosis of differentiated hepatocytes caused by Bcl-xL deficiency. This is surprising, because differentiated hepatocytes are generally considered to be quiescent cells. Organ homeostasis may be ensured in two ways: one is through turnover of cells, and the other is by the quies-

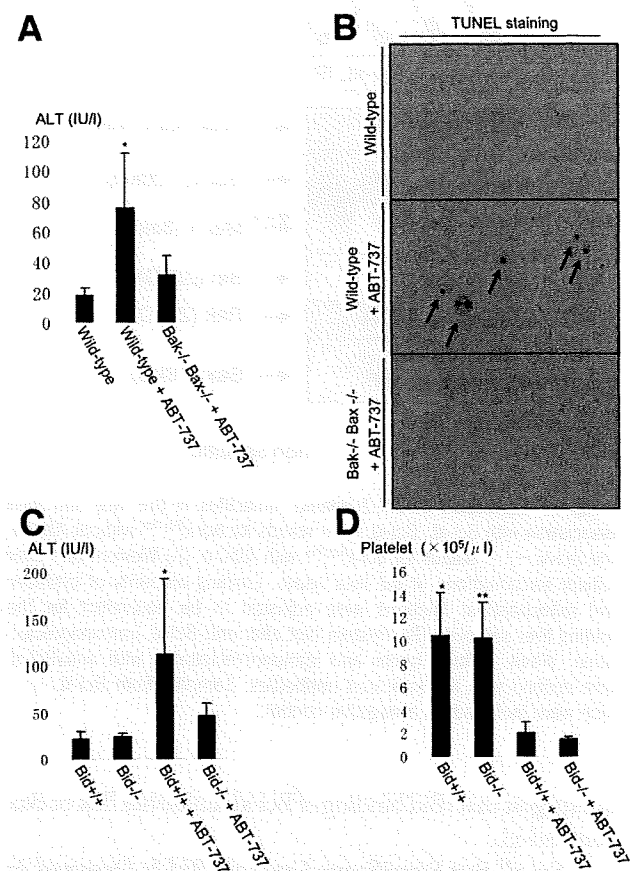


Fig. 6. ABT-737 administration in wild-type, Bak/Bax double-KO, and Bid KO mice. (A and B) Wild-type mice or hepatocyte-specific Bak/Bax double-KO mice were challenged with intraperitoneal injection of ABT-737 at 100 mg/kg or vehicle alone and sacrificed 16 hours later. Serum ALT levels (A) and representative pictures of TUNEL staining in the liver (B) are shown. N = 5 or more than 5 mice per group. **P* < 0.05 versus the other two groups. (C and D) Wild-type mice or Bid KO mice were challenged with intraperitoneal injection of ABT-737 at 100 mg/kg or vehicle alone and sacrificed 16 hours later. Serum ALT levels (C) and circulating platelet counts (D) were determined. N = 5 or more than 5 mice per group. **P* < 0.05 versus the other three groups for (C); * and ***P* < 0.05 versus the other two groups, with ABT-737 for (D).

cence of matured cells. Typical examples for the former are hematopoietic organs, intestine and skin, whereas those for the latter are a variety of solid organs, such as the liver, lung, pancreas, heart, and brain. Because hematopoietic cells die at particular time points to maintain host homeostasis, it would not be surprising that their life span may be controlled by a variety of death signals. Indeed, Bim KO mice have excess hematopoietic cells, particularly lymphocytes, suggesting that Bim strictly controls homeostasis of hematopoietic cells.²³ In contrast, healthy cells in the solid organs are usually considered to not suffer from apoptotic stimuli. Although interaction between core Bcl-2 proteins and BH3-only proteins is important for understanding apoptosis regulation, little work has been done by generating mice simultaneously deficient in molecules of both groups. To the best of our knowledge, the only example clearly using this approach is a study on Bim/Bcl-2 double-KO mice that showed that growth retardation, skin abnormality, and lymphoid cell reduction found in Bcl-2 KO mice were ameliorated with a Bim-deficient background.²⁴ This suggested that lymphoid cells constitutively sense Bim-mediated killing signals, and, without Bcl-2, decrease in number. The current study is the first demonstration that parenchymal cells in a solid organ such as differentiated hepatocytes also suffer from Bid-mediated BH3 stress.

Bid is ubiquitously expressed in many cell types. Generally, Bid is inactive for death induction and is activated on proteolytic cleavage by caspase-8 or other proteases. In the current study, we found that not only full-length Bid but also tBid could be detected in wild-type liver. Administration of tBid, but not that of full-length Bid, at 20 nM in wild-type liver lysate or mitochondria was sufficient for unleashing Bak or Bax from Bcl-xL and releasing cytochrome c. Conversely, a lesser amount of tBid (for example, at 2 nM) was sufficient for inducing cytochrome c release from Bcl-xL-deficient mitochondria. These results are consistent with the idea that a small amount of tBid produced in the liver could activate cytochrome c release and apoptosis in hepatocytes of the Bcl-xL-deficient mice. What mechanisms are involved in the production of tBid from full-length Bid in the healthy liver is not known yet. Our results suggest that Myd88 and TNF- α may not be involved in the activation of tBid under physiological conditions. However, other ligation of death receptors such as Fas, and TNF-related apoptosis-inducing ligand receptor, can cause caspase-8 activation followed by Bid cleavage.^{15,25} Bile salts, which are consistently produced in and secreted from hepatocytes, are capable of inducing hepatocyte apoptosis through Fas activation.²⁶ Natural killer cells are predominant lymphocytes accumulating in the liver and constitutively express TNF-re-

lated apoptosis-inducing ligand.²⁷ Further study is needed to examine what kinds of stresses activate the Bid pathway in a physiological setting.

Adult differentiated hepatocytes express at least two anti-apoptotic Bcl-2 proteins, Bcl-xL and Mcl-1, but not prototype Bcl-2.⁸ Recently, Vick et al.²⁸ reported that hepatocyte-specific Mcl-1 KO mice developed naturally occurring apoptosis in hepatocytes. We also independently generated hepatocyte-specific Mcl-1 KO mice and obtained an apoptosis phenotype that could not be distinguished from that of hepatocyte-specific Bcl-xL KO mice.²⁹ Thus, Mcl-1, like Bcl-xL, plays a critical role in maintaining integrity of differentiated hepatocytes. There are two major models regarding how BH3-only proteins mediate Bak/Bax-dependent apoptosis: a direct model and an indirect model.³⁰ From the viewpoint of the indirect model, our data would mean a small amount of tBid is sequestered by Bcl-xL and Mcl-1 and, without Bcl-xL, is sufficient for neutralizing Mcl-1 to promote apoptosis. Conversely, from the viewpoint of the direct model, both Bcl-xL and Mcl-1 are needed to completely sequester a small amount of tBid, and without Bcl-xL, unleashed tBid would directly activate Bak and Bax. In the current study, we observed that tBid when administered in liver lysate could bind to Bcl-xL. This observation seems to agree with the indirect model, although we could not exclude the possibility of the direct model. Further study will be needed by developing Bid/Bcl-xL/Mcl-1 KO mice to examine the underlying mechanisms of how activated Bid regulates the mitochondrial pathway of apoptosis in the liver.

Malignant tumors frequently overexpress one or more members of the anti-apoptotic Bcl-2 family, which confers the resistance of tumor cells to apoptosis.^{31,32} Recently, small molecules targeting specific anti-apoptotic Bcl-2 family proteins have been developed for treatment of cancer therapy.^{33,34} The underlying concept of this strategy is the difference in addiction to anti-apoptotic Bcl-2 family proteins between normal cells and transformed cells. In general, normal cells are not considered to suffer from apoptotic stimuli or to have activated BH3-only proteins. In contrast, transformed cells suffer from a variety of apoptotic stimuli such as genotoxic *p53* activation and environmental stresses, and possess activated BH3-only molecules. If a single anti-apoptotic Bcl-2 protein is neutralized by a small molecule, it could release BH3-only molecules, which then neutralize other anti-apoptotic Bcl-2 proteins or directly activate Bax-like molecules, leading to cell death. However, the current study clearly indicated that normal hepatocytes could be under activation of Bid, raising concern that hepatocyte injury may be produced if Bcl-xL function is completely knocked down. Indeed, we have shown that administra-

tion of a high dose of ABT-737, which is an antagonist for Bcl-xL/Bcl-2, not for Mcl-1,²¹ induced Bak/Bax-dependent hepatocyte apoptosis in wild-type mice but to a lesser extent in Bid KO mice. Therefore, special caution should be paid to hepatotoxicity when systemically administering a high dose of Bcl-xL-targeting molecules, because hepatocytes are suffering from Bid-mediated stresses.

In conclusion, we have demonstrated here that the BH3-only protein Bid is activated and antagonized by anti-apoptotic Bcl-2 family proteins under physiological conditions. BH3 stress or Bcl-2 addiction is not a unique characteristic of tumor cells. Even in healthy cells, cellular integrity is not controlled by a simple rheostat between Bax-like molecules and Bcl-2-like molecules. The current study reveals a previously unrecognized complicated network of Bcl-2 family proteins controlling the integrity of healthy cells. Dissection of the Bcl-2 network will be important for further understanding of liver pathophysiology.

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Natural killer cell is a major producer of interferon γ that is critical for the IL-12-induced anti-tumor effect in mice

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Abstract Although the anti-tumor effect of IL-12 is mediated mostly by IFN γ , which cell types most efficiently produce IFN γ and therefore initiate or promote the anti-tumor effect of IL-12 has not been clearly determined. In the present study, we demonstrated hydrodynamic injection of the IL-12 gene led to prolonged IFN γ production, NK-cell activation and complete inhibition of liver metastasis of CT-26 colon cancer cells in wild-type mice, but not in IFN γ knockout mice. NK cells expressed higher levels of STAT4 and upon IL-12 administration displayed stronger STAT4 phosphorylation and IFN γ production than non-NK cells. Adoptive transfer of wild-type NK cells into IFN γ knockout mice restored IL-12-induced IFN γ production, NK-cell activation and anti-tumor effect, whereas transfer of the same number of wild-type non-NK cells did not. In conclusion, NK cells are predominant producers of IFN γ that is critical for IL-12 anti-tumor therapy.

Keywords IFN γ · Innate immunity · Liver tumor · IL-12 · NK

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Introduction

IL-12 is a 70-kDa heterodimer protein, composed of p35 and p40 subunits, mainly produced by antigen-presenting cells. IL-12 was originally found as a “natural killer-stimulating factor” and a “cytotoxic lymphocyte maturation factor” [1, 2]. IL-12 has multi-potent effects, inducing a Th1 response, enhancing the CD8 T-cell response, activating natural killer cells and inducing production of IFN γ [3, 4]. Therapeutic use of IL-12, either using its recombinant protein or gene, can induce an efficient anti-tumor effect on primary or metastatic tumors in various murine models and humans [5, 6].

Research has shown that IL-12 mediates anti-tumor effects in a variety of ways. They include anti-proliferative effects, anti-angiogenic effects [7, 8] and cytotoxic effects of effector lymphocytes. A variety of effector cells has been reported to be required for IL-12-mediated anti-tumor effects: they include CD8 T cells [9], NKT cells [10], CD4 T cells [11] and NK cells [12]. The relative contribution of these cells may differ among IL-12 doses and types of tumor models [13]. Endogenous IFN γ production is required for most, if not all, of the anti-tumor effects of IL-12 administration [14, 15]. IL-12 stimulates a variety of immune cells, such as T cells [16], B cells [17] and NK cells [18], to produce IFN γ . However, which cell types are most critical for producing IFN γ during IL-12 therapy is not clearly known.

In the present study, we used a murine model of liver metastasis of CT-26 colon cancer cells and found that NK cells highly expressed the IL-12 signaling molecule STAT4 and most efficiently produced IFN γ . IFN γ was essential for the anti-tumor effect of IL-12, and NK-cell production of IFN γ sufficed to produce the full-blown anti-tumor effects. These results demonstrated that NK cells

serve not only as an effector but also as an important mediator producing IFN γ that is critical for the anti-tumor effects of IL-12.

Materials and methods

Mice

Specific pathogen-free female Balb/c mice were purchased from Clea Japan, Inc (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice with a Balb/c background were purchased from Taconic (Germantown, NY). IFN γ knockout (GKO) mice with a Balb/c background were kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo). All mice used were at the age of 6 to 10 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intra-splenic injection of tumor cells was used to establish micro-disseminated liver tumors in mice [19]. CT-26 colon cancer cells originating from Balb/c mice were maintained in RPMI1620 supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 200 μ l of PBS and injected into the spleen.

Injection of naked plasmid DNA

A plasmid coding the murine IL-12 gene, pCMV-IL-12, was generously provided by Dr. M Watanabe (Laboratory of Experimental Immunology, Division of Basic Sciences, National Cancer Institute-Frederick Cancer Research and Development Center) [20]. Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany,) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described [21]. In brief, 25 μ g of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 26-gauge needle. DNA injection was completed within 5 to 8 s.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels

of serum IL-12 p70, IFN γ (BD Biosciences-Pharmingen, San Diego, CA), IFN γ -inducible protein 10 (IP-10) and monokine induced by IFN γ (MIG) (R&D Systems, Inc, Minneapolis, MN) were measured using commercially available ELISA kits in accordance with the manufacturer's instructions.

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described. The NK activity of mononuclear cells was assessed by a standard 4-h ^{51}Cr -releasing assay using Yac1 cells as targets. In some experiments, mononuclear cells were separated into DX5 $^{+}$ cells (NK cells) and DX5 $^{-}$ cells (non-NK cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated NK-cell population was found to be greater than 90% by FACS analysis.

Flow cytometric analysis

Liver mononuclear cells were isolated 2 days after pCMV-IL-12 injection. Cytokine secretion was then blocked by the addition of brefeldin A for 4 h. Next, liver mononuclear cells were stained with FITC-conjugated anti-TCR β antibody and biotin-conjugated anti-CD49b antibody (DX5), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with PE-conjugated anti-IFN γ antibody or corresponding isotype controls. Analysis was performed using a FACSCalibur (Becton Dickinson), with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as DX5 $^{+}$ /TCR β $^{-}$ lymphocytes, NKT cells as DX5 $^{+}$ /TCR β $^{+}$ lymphocytes and T cells as DX5 $^{-}$ /TCR β $^{+}$ lymphocytes.

Adoptive transfer

For adoptive transfer experiments, GKO mice were injected intravenously 1 day before plasmid DNA injection with 2.0×10^8 whole mononuclear cells or 4.0×10^6 NK cells, or non-NK cells or whole mononuclear cells, all of which had been harvested from wild-type mice that can produce IFN γ .

Western blotting

Mouse recombinant IL-12 was purchased from R&D Systems, Inc (Minneapolis, MN). Mononuclear cells were treated with or without IL-12. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μ g of protein was separated by SDS-PAGE and transferred to the PVDF membrane. The membrane was stained with anti-STAT4 antibody (BD biosciences),

anti-phospho-specific STAT4 (pY693) antibody (BD Biosciences), anti-STAT1 antibody (Cell Signaling), anti-phospho-specific STAT1 antibody (Cell Signaling) and visualized by chemiluminescence.

NK-cell depletion

For depletion of NK cells *in vivo*, anti-asialoGM1 antibody (WAKO, Osaka, Japan) was intraperitoneally administered. We determined the appropriate dosing to be 500 $\mu\text{g}/\text{mouse}$ (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. The percentage of $\text{DX5}^+/\text{TCR}\beta^-$ cells (NK cells) is $12.6 \pm 2.4\%$ in IgG-injected liver, whereas it decreased to $0.76 \pm 0.04\%$ one day after anti-asialo GM1 antibody injection ($N = 3/\text{group}$). This effect remained at least 3 days after anti-asialo GM1 antibody injection. NKT cells were less affected than NK cells, because 90% of $\text{DX5}^+/\text{TCR}\beta^+$ cells (NKT cells) still remained in the liver after the treatment. Anti-asialoGM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Histology

The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Acetone-fixed fresh frozen liver sections were immunostained with anti-mouse CD4 (H123.19), anti-mouse CD8 α (53-6.7) or anti-CD31 (390) monoclonal antibody (all from BD Biosciences), using a VECSTAIN ABC kit (Vector Laboratories, Burlingame, California, USA).

Statistics

Data are represented as mean \pm SD. Comparisons between groups were analyzed by unpaired *t*-test with Welch's correction. $p < 0.05$ was considered statistically significant.

Results

Hydrodynamic injection of IL-12-expressing plasmid led to prolonged production of IFN γ

Hydrodynamics-based gene delivery into mice establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes. Serial measurement of serum IL-12 demonstrated that pCMV-IL-12 injection led to substantial IL-12 production on day 1. The levels of

serum IL-12 then rapidly declined (Fig. 1a). We also measured IFN γ production in serum, since IL-12 is known to activate IFN γ production. pCMV-IL-12 and, to a lesser extent, pCMV injection increased serum IFN γ on day 1. In contrast to the pCMV injection group, high levels of serum IFN γ were maintained at later time points in the pCMV-IL-12 injection group (Fig. 1a). Thus, hydrodynamic injection of pCMV-IL-12 led to prolonged production of IFN γ . Transient IFN γ production followed by control plasmid may be an indirect effect of liver injury caused by bolus injection of saline or DNA injection.

IL-12 therapy induced NK activation and anti-metastatic effects, both of which are critically dependent on IFN γ

To examine the biological effects of the produced IL-12, we evaluated the NK activity of mononuclear cells from the liver. pCMV-IL-12 injection, but not control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells (Fig. 1b). When GKO mice were injected with pCMV-IL-12 or pCMV, the hepatic mononuclear cells did not display any lytic ability to Yac1 cells, suggesting that IL-12-mediated NK-cell activation required IFN γ .

To examine the anti-metastatic effect of IL-12, pCMV-IL-12 or pCMV was injected into wild-type mice 2 days after intrasplenic injection of CT-26 cells. At 14 days after tumor injection, the mice were killed for evaluation of liver tumor (Fig. 1c). While pCMV-injected mice displayed huge liver tumors, pCMV-IL-12-injected mice did not show any macroscopic or microscopic tumor (Fig. 1d). Liver weight was significantly higher in pCMV-injected mice than pCMV-IL-12-injected mice, reflecting liver tumor formation. To examine the involvement of IFN γ in the IL-12-induced anti-tumor effect, we injected pCMV or pCMV-IL-12 into GKO mice 2 days after CT-26 injection. At 14 days after CT-26 injection, both groups showed similar degrees of tumor formation and there was no significant difference in liver weight between the two. This indicated that IL-12-induced anti-metastatic effect was strictly dependent on IFN γ .

NK cells were the most potent producer of IFN γ during IL-12 therapy

To evaluate which cell types most efficiently produced IFN γ , we isolated hepatic mononuclear cells from mice 2 days after plasmid injection and then stained cell surface TCR β and DX5 as well as intracellular IFN γ (Fig. 2). TCR $\beta^-/\text{DX5}^+$ NK cells, TCR $\beta^+/\text{DX5}^+$ NKT cells and TCR $\beta^+/\text{DX5}^-$ T cells from pCMV-IL-12-injected mice showed significant levels of IFN γ production compared

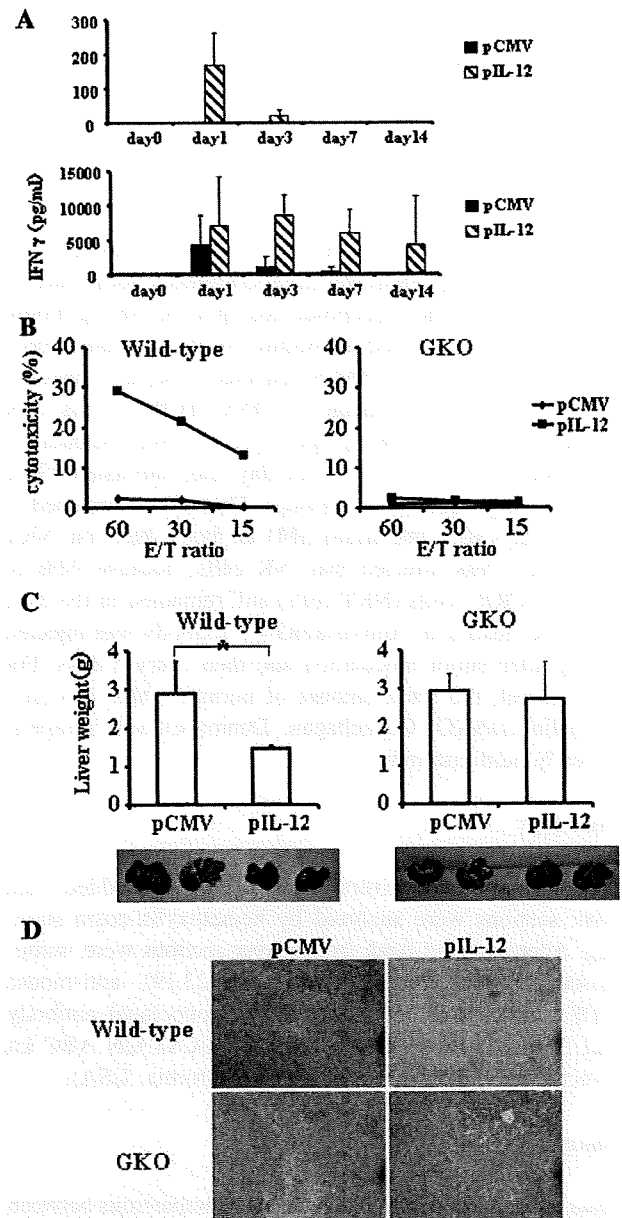
Fig. 1 Effects of hydrodynamic injection of IL-12-encoding plasmid. **a** Wild-type mice were hydrodynamically injected with either pCMV-IL-12 (hatched bars) or pCMV (closed bars) and bled at the indicated time points to measure the levels of serum IL-12 and IFN γ . Results are indicated as mean and SD ($n = 6$ /group). **b** NK-cell activation after IL-12 administration. Hepatic mononuclear cells were isolated from wild-type mice (left) or GKO mice (right) which had been injected with pCMV-IL-12 (closed squares) or pCMV (closed diamonds) 4 days earlier. Yac1 lytic ability was measured by a standard ^{51}Cr -release assay at the indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown. **c** and **d** Anti-metastatic effects of IL-12 therapy. Wild-type mice (left) or GKO mice (right) were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. At 14 days after the plasmid injection, the mice were killed to examine liver tumor development. **c** Data are indicated as mean and SD of the liver weight at the top ($n = 6$ /group) and a representative picture of the liver in each group is shown at the bottom. * $p < 0.001$. **d** Representative histology of liver sections

with those from naive mice or pCMV-injected mice. The levels of IFN γ production were highest in NK cells among those cells. Even at a later time point, 7 days after plasmid injection, NK cells were found to produce the highest levels of IFN γ (data not shown).

IL-12-induced STAT4 signaling and IFN γ production increased in NK cells

IL-12 activates Janus kinases Tyk2 and Jak2, STAT4 as well as other STATs. To examine the activation of STAT1 and STAT4, we isolated splenocytes from wild-type mice and GKO mice and stimulated them with IL-12 and/or IFN γ in the presence or absence of anti-IFN γ Ab (Fig. 3a). IL-12 led to phosphorylation of both STAT1 and STAT4 in wild-type splenocytes. In contrast, the same treatment led to phosphorylation of STAT4, but not of STAT1, in GKO splenocytes. Addition of IFN γ restored STAT1 phosphorylation in GKO splenocytes. Furthermore, adding anti-IFN γ inhibited STAT1 phosphorylation in wild-type cells. These findings demonstrated that phosphorylation of STAT4 is a direct effect of IL-12 but phosphorylation of STAT1 is indirect, via an autocrine or paracrine IFN γ -dependent manner.

To examine STAT1 and STAT4 activation and IFN γ production in NK cells and non-NK cells, we prepared whole mononuclear cells as well as NK and non-NK populations from wild-type spleens and stimulated the cells with IL-12 (Fig. 3b). NK cells expressed higher levels of STAT4 than non-NK cells. Upon IL-12 treatment, STAT4 was rapidly phosphorylated in NK cells, but to a lesser extent in non-NK cells. In contrast, NK cells expressed lesser levels of STAT1 than non-NK cells. STAT1 was similarly phosphorylated in NK cells and non-NK cells upon IL-12 treatment. Both NK cells and non-NK cells



produced significant levels of IFN γ , but the levels were much higher in NK cells than non-NK cells (Fig. 3c). These results indicated that compared with non-NK cells, NK cells possessed higher levels of STAT4, a direct signaling molecule of IL-12, and produced higher levels of IFN γ than non-NK cells.

NK cells were sufficient for IL-12-mediated anti-tumor effects

The above observation indicated that NK cells are a predominant producer of IFN γ , which was critical for the IL-12-induced anti-tumor effects. To examine whether NK

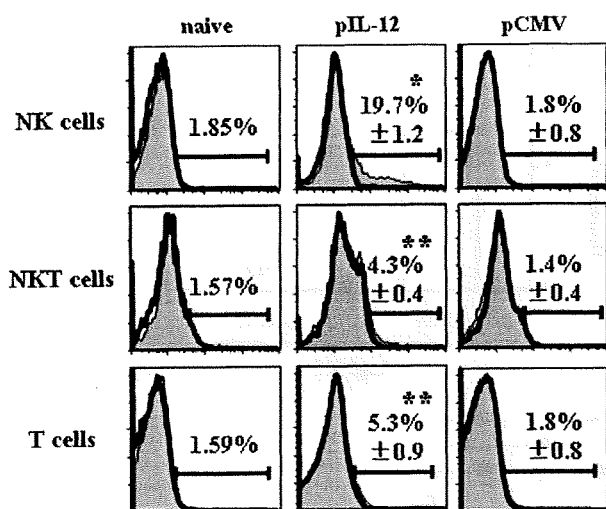


Fig. 2 IFN γ expression of mononuclear cells after IL-12 administration. Wild-type mice were injected with pCMV-IL-12 or pCMV, or were untreated (naive). Mononuclear cells were isolated from the liver 2 days after plasmid injection and stained with anti-TCR β mAb, anti-DX5 mAb and anti-IFN γ mAb. Closed histograms show the IFN γ expression in the gated populations (TCR β /DX5 $^{+}$ cells for NK cells, TCR β^{+} /DX5 $^{+}$ cells for NKT cells and TCR β^{+} /DX5 $^{-}$ cells for T cells). Isotype control stainings are shown by open histograms. Numbers in histograms represent averages \pm SD of percentages of positive cells ($n = 3$ mice/group). * $p < 0.0001$ vs. mock in NK populations. ** $p < 0.05$ vs. mock in each population

cells are sufficient for the anti-metastatic effects of IL-12, we examined the anti-metastatic effect in Rag2 KO mice which lack T cells, B cells and NKT cells. pCMV-IL-12 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in Rag2 KO mice higher than in wild-type mice (Fig. 4a). To examine whether NK cells are sufficient for IL-12-mediated rejection of hepatic metastasis, we injected pCMV-IL-12 or pCMV into mice that had been intra-splenically injected with CT-26 cells 2 days earlier. Serum IFN γ levels of Rag2 KO mice were about 4 times higher than those of wild-type mice (Fig. 4b). pCMV-IL-12 completely suppressed hepatic metastasis in Rag2 KO mice (Fig. 4c).

Adoptive transfer of wild-type NK cells into GKO mice restored the anti-tumor effects of IL-12

Since NK cells were sufficient for producing IL-12-induced anti-tumor effects, we postulated that their production of IFN γ may play an important role in these effects. To test this, we performed adoptive transfer experiments with GKO mice. First, whole mononuclear cells isolated from the spleens of wild-type mice (2.0×10^8 cells) were adoptively transferred to GKO mice 1 day before plasmid injection. pCMV-IL-12 injection increased Yac1 lytic activity of hepatic mononuclear cells in the adoptively

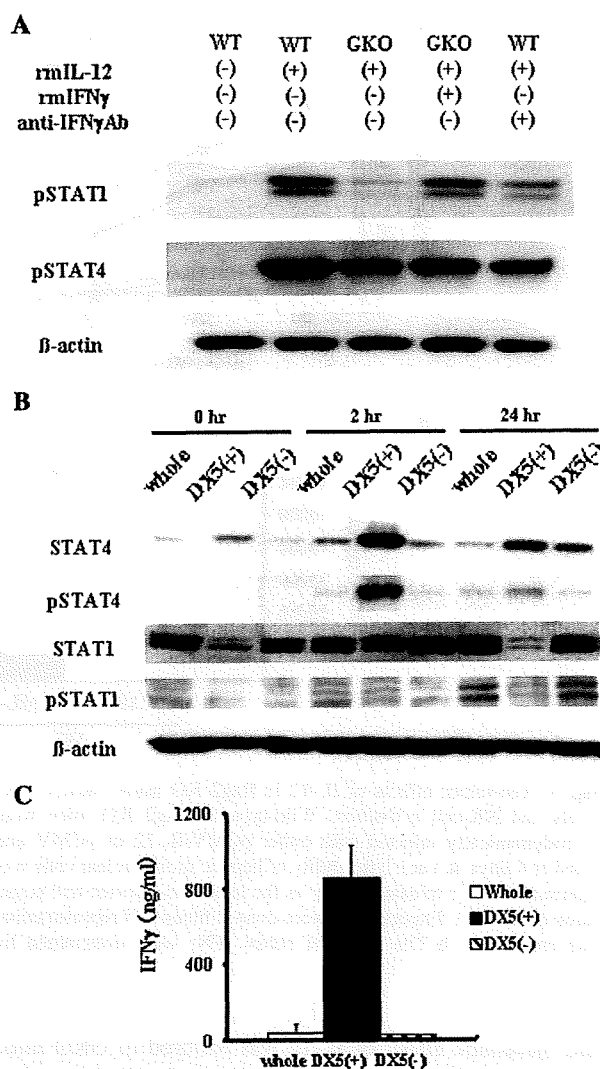


Fig. 3 STAT signaling and IFN γ production of mononuclear cells in vitro treated with IL-12. **a** STAT1 and STAT4 activation of splenocytes in vitro treated with IL-12. Splenocytes were isolated from wild-type mice or GKO mice and treated with or without recombinant IL-12 (20 ng/mL) in the presence or absence of recombinant IFN γ (500 ng/mL) or anti-IFN γ antibody (20 μ g/mL) for 24 h. Cellular lysates were analyzed by Western blot for the expression of phospho-STAT1, phospho-STAT4 and β -actin. **b** and **c** STATs expression and signaling of NK cells and non-NK cells. Splenocytes were isolated from wild-type mice. Whole splenocytes were further purified into DX5 $^{+}$ cells and DX5 $^{-}$ cells. Each cell population was cultured with recombinant IL-12 (20 ng/mL) for the indicated times. **b** The cells were lysed to examine expression of whole STAT and phospho-STAT by Western blot. **c** The levels of IFN γ in the culture supernatant at 24 h were determined by ELISA. Data are expressed as mean and SD ($n = 3$)

transferred group, but not in the untreated group (Fig. 5a). pCMV-IL-12 induced significant increase in serum IFN γ levels 4 days after plasmid injection in the adoptive transferred group, but not in the other groups (Fig. 5b). The

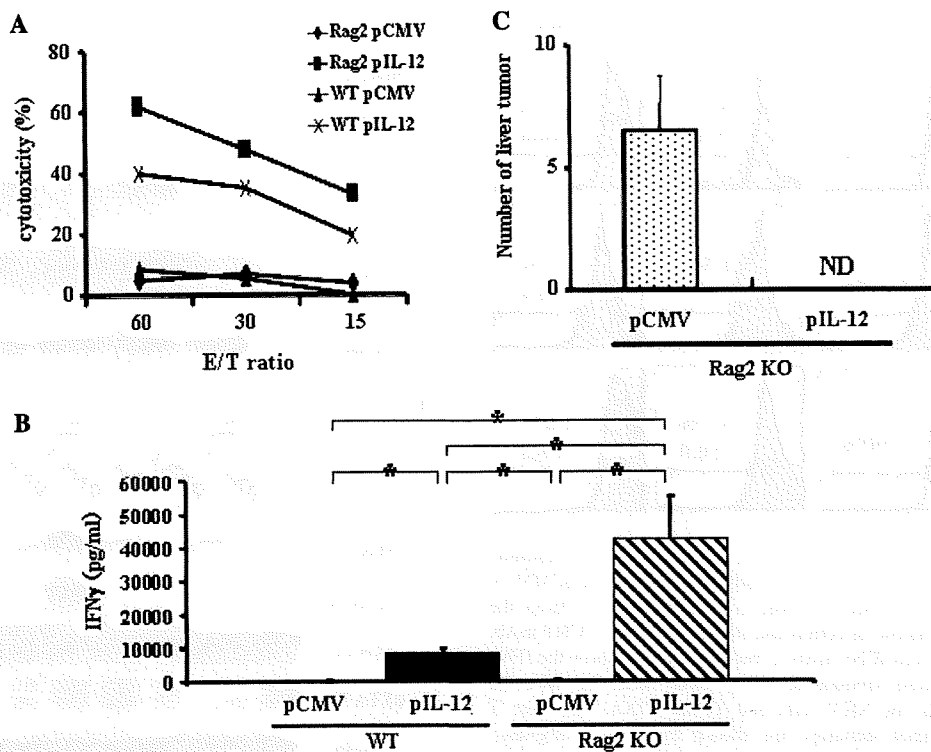


Fig. 4 Anti-tumor effects of IL-12 in Rag2 KO mice. Serum IFN γ levels and NK-cell activation. Wild-type or Rag2 KO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV and killed at 4 days. **a** Yac1 lytic ability of hepatic mononuclear cells was determined by Cr releasing assay as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFN γ were determined by

ELISA. Data are expressed as mean and SD ($n = 7/\text{group}$). $*p < 0.0001$. **c** Anti-metastatic effect. Rag2 KO mice were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Fourteen days after plasmid injection, mice were killed to examine tumor development in the liver. The numbers of hepatic tumors in each group are expressed as mean and SD ($n = 7/\text{group}$). ND not detectable

anti-metastatic effect of IL-12 was restored in GKO mice when whole mononuclear cells from wild-type mice were adoptively transferred (Fig. 5c).

To evaluate the contribution of IFN γ production from each subset of mononuclear cells to the anti-metastatic effect of IL-12, we adoptively transferred the same number of whole mononuclear cells, NK cells or non-NK cells from wild-type mice (4.0×10^6 cells) 1 day before pCMV-IL-12 injection and analyzed liver tumor formation. Only in the NK-cell-transferred group, pCMV-IL-12 injection induced NK cytolytic ability in the liver and IFN γ elevation in serum 4 days after plasmid injection, but not in the other groups (Fig. 5d, e). No liver tumor formed in the NK-cell-transferred group. In contrast, livers in other groups had massive tumors, and the liver weights were significantly heavier than those in the NK-cell-transferred group (Fig. 5f). These results clearly demonstrated the strong impact of IFN γ produced from NK cells on IL-12-induced anti-tumor effects compared with that from non-NK cells.

Anti-tumor effects of IL-12 deteriorated slightly in mice depleted of NK cells

To examine the involvement of NK cells in the tumor deletion by IL-12 therapy, we induced depletion of NK cells by repeatedly injecting anti-asialoGM1 antibody. The cytolytic ability of NK cells was completely abolished in the anti-asialoGM1 antibody-injected group (Fig. 6a). Serum IFN γ induction by IL-12 in the NK depletion group was about half of that in the control immunoglobulin injected group (Fig. 6b). Unexpectedly, pCMV-IL-12 injection inhibited macroscopic liver metastasis of CT-26 cells in NK cell-depleted mice (Fig. 6c). However, a number of microscopic tumor regions were observed after IL-12 therapy in NK cell-depleted mice but not in control IgG-injected mice (Fig. 6d). This finding indicated that NK cells are required for a full-blown IL-12 anti-tumor effect, but IL-12's anti-tumor effect was still observed even if the NK cells were knocked down. To examine the underlying mechanisms of anti-tumor effect in NK cell-depleted mice,

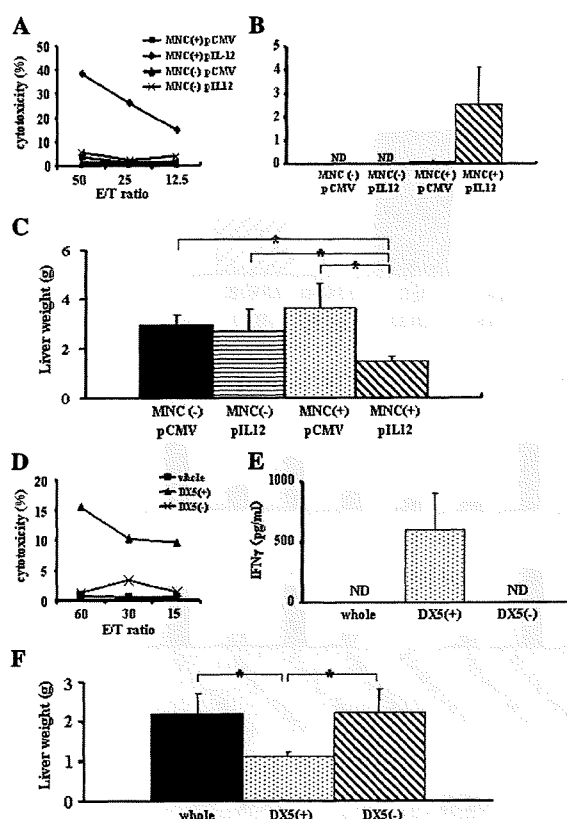


Fig. 5 Adoptive transfer of wild-type cells into GKO mice. Adoptive transfer of wild-type splenocytes restored anti-tumor effects of IL-12 in GKO mice. **a** GKO mice were intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. Yac1 lytic ability of hepatic mononuclear cells was expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **b** and **c** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice. Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **b** The levels of serum IFN γ 4 days after plasmid injection are expressed as mean and SD ($n = 6$ /group). **c** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are indicated as mean and SD ($n = 6$ /group). ND not detectable. $*p < 0.01$. Adoptive transfer of wild-type-NK cells, but not non-NK cells, restored anti-tumor effects of IL-12 in GKO mice. **d** Wild-type splenocytes were purified into DX5 $^+$ cells and DX5 $^-$ cells. GKO mice were intravenously injected with 4.0×10^6 whole mononuclear cells or DX5 $^+$ cells or DX5 $^-$ cells and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after hydrodynamic injection. Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **e** and **f** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with whole mononuclear cells, DX5 $^+$ cells or DX5 $^-$ cells (4.0×10^6 /mouse). Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **e** The levels of serum IFN γ are expressed as mean and SD ($n = 6$ /group). **f** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are expressed as mean and SD ($n = 6$ /group). ND not detectable. $*p < 0.001$

serum levels of IP-10 and MIG, chemokines downstream of IFN γ , were measured after IL-12 therapy (Fig. 6e). pCMV-IL-12-injected mice showed significant increase in both levels compared with pCMV-injected mice. Significant increase after pCMV-IL-12 injection was also found in NK cell-depleted mice, but not in GKO mice. This result suggests that production of these chemokines was not completely suppressed in NK cell-depleted mice in our experimental condition. Immunohistochemical analysis revealed that tumoral accumulation of CD4-positive cells and CD8-positive cells was observed in pCMV-IL-12-injected mice but not in pCMV-injected mice. On the other hand, similar levels of CD31 expression were observed in tumors of pCMV-injected mice and pCMV-IL-12-injected mice (Fig. 6d). These results suggest that IL-12's anti-tumor effects might be mediated by T-cell accumulating in the tumor rather than anti-angiogenesis.

Discussion

IL-12 is recognized as a master regulator of adaptive type 1, cell-mediated immunity. One major action of IL-12 is its induction of other cytokines, particularly IFN γ . A large amount of evidence has indicated that IL-12 administration leads to IFN γ production from a variety of immune cells, such as T cells [16], B cells [17], NK cells [18] and NKT cells [22]. The relative impact of each immune cell as the source of IFN γ has been controversial. The present study highlighted NK cells as a most efficient producer of IFN γ that is critical for IL-12-induced anti-tumor effects.

Flow cytometric analysis revealed higher *in vivo* production of IFN γ of NK cells than that of other cell types. The levels of serum IFN γ were around fourfold higher in Rag2 KO mice which only possess NK cells than in wild-type mice. On the other hand, NK-cell depletion in wild-type mice led to twofold reduction of serum IFN γ levels. These data indicate substantial contribution of NK cells in IFN γ production *in vivo*. Previous research has demonstrated that the specific cellular effects of IL-12 are due mainly to activation of STAT4 [23, 24]. IL-12-induced STAT4 phosphorylation leads to the production of IFN γ [25]. In agreement with these reports, our *in vitro* analysis showed that, in contrast to STAT1, STAT4 was directly phosphorylated upon IL-12 stimulation, being independent of IFN γ . Of interest is the finding that NK cells express higher levels of STAT4 than non-NK cells, suggesting that NK cells possess an ideal expression profile of STATs for producing IFN γ upon IL-12 stimulation. Indeed, *in vitro* analysis revealed that NK cells, upon IL-12 exposure, displayed higher levels of IFN γ production as well as STAT4 phosphorylation than non-NK cells. These *in vitro*

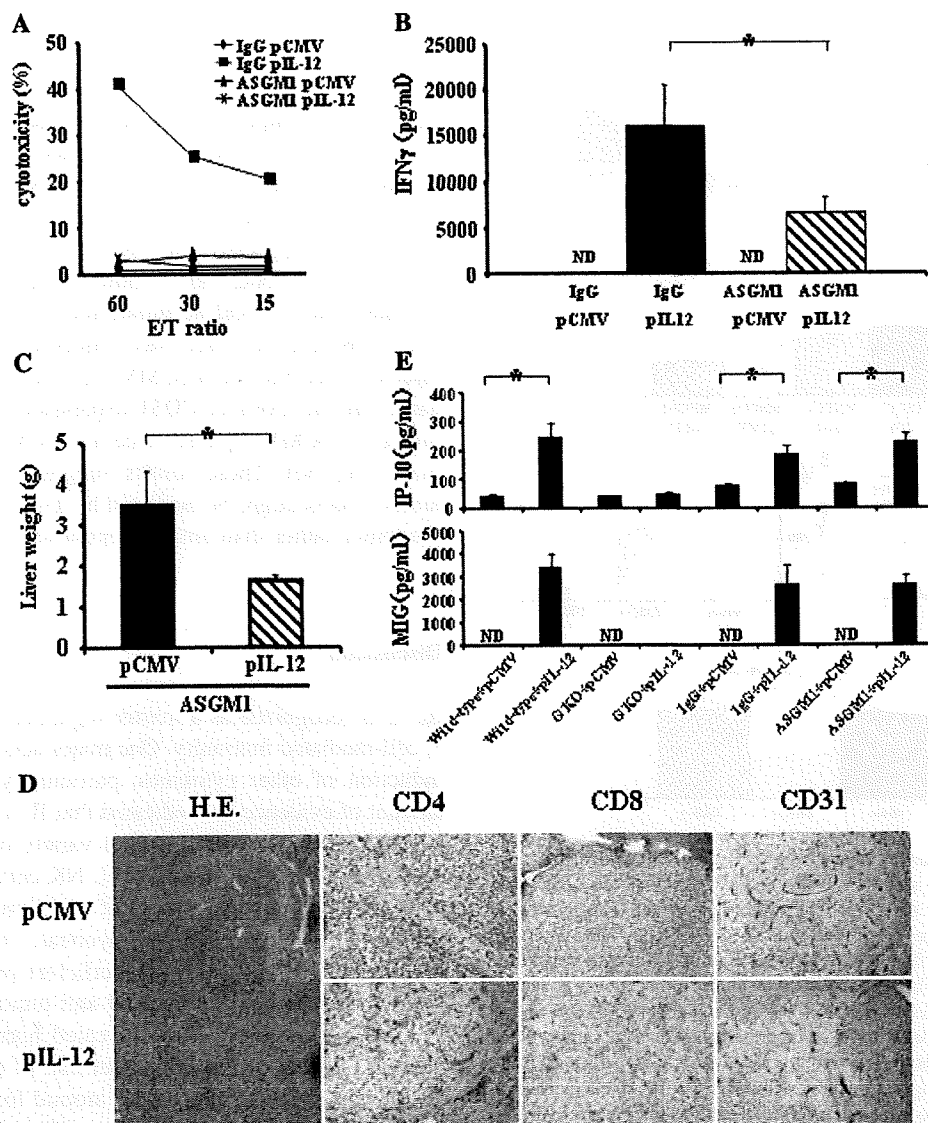


Fig. 6 Anti-tumor effects of IL-12 in NK-cell-depleted mice. Serum IFN γ levels and NK-cell activation. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and, 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. **a** Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFN γ are expressed as mean and SD ($n = 6$ /group). ND not detectable. $*p < 0.005$. Anti-metastatic effects. Wild-type mice were intrasplenically injected with CT-26 cells and, 1 day later and then every 5 days, intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and hydrodynamically injected with either pCMV-IL-12 or pCMV 2 days after CT-26

injection. Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. **c** The results are indicated as mean and SD ($n = 6$ /group). $*p < 0.001$. **d** Representative histology of liver sections analyzed by hematoxylin-eosin staining and immunohistochemistry of CD4, CD8 and CD31. **e** Serum levels of IP-10 and MIG. Wild-type or GKO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Four days later, each mice were bled to measure the levels of serum IP-10 and MIG. Results are expressed as mean and SD ($n = 6$ /group). ND not detectable. $*p < 0.001$

data are consistent with the in vivo observation that NK cells are efficient producers of IFN γ during IL-12 therapy.

Many studies have demonstrated that IFN γ production is required for the anti-tumor effects of IL-12 [14, 26, 27]. In fact, we have demonstrated that deletion of IFN γ abolished

NK cytotoxicity and the anti-metastatic effect of IL-12 therapy in the liver. A large amount of evidence supports the concept that a major action of IL-12 is to promote the differentiation of naïve CD4 + T cells into Th1 cells, which produce IFN γ . Previous research reported that CD4

T-cell depletion caused inhibition of anti-tumor effects. More recent studies have supported a critical role of IFN γ as a third signal for CD8 T-cell differentiation. There have been many reports focusing on IFN γ production from T cells induced by IL-12 for the anti-tumor effect of IL-12 [28]. Segal et al. performed an elegant study showing a critical role of T-cell production of IFN γ in the anti-tumor effect by adoptively transferring T cells into GKO mice in a subcutaneous tumor model [29]. However, apart from this study, little is known about the contribution of each immune cell as a producer of IFN γ in terms of an anti-tumor effect. In our model, T-cell mediated adaptive responses were not required for the anti-metastatic effect of IL-12. More importantly, the anti-metastatic effects of IL-12 were restored in GKO mice by an adoptive transfer of wild-type NK cells. The same number of non-NK cells could not provoke IL-12-induced anti-tumor effects in GKO mice. The present study demonstrated for the first time a potent effect of NK cells on producing IFN γ that was critical for anti-metastatic effect during IL-12 therapy.

Our study showed that the main IFN γ producer of IL-12 was NK cells. So we focused on NK cells which were activated by IL-12 in an IFN γ -dependent manner to examine the cellular mechanism of protection against hepatic metastasis. Many studies have shown the importance of each subset (NK- [12], NKT- [10] and T [9, 30] cells) for anti-tumor effects of IL-12. In the present study, NK cells were sufficient while T cells, B cells, NKT cells were dispensable for IL-12-mediated NK-cell activation and anti-metastatic effects as IL-12 therapy showed Yac1 lytic ability and antimetastatic effects in Rag2 KO mice. On the other hand, NK-cell depletion by a repeated injection of anti- α GalGMI antibody protected wild-type mice from macroscopic liver metastasis, but did not from microscopic liver metastasis. Thus, although NK cells were required for a full-blown IL-12 anti-tumor effect, other anti-tumor pathways are activated by IL-12 in the absence of NK cells. Serum levels of IP-10 and MIG suggest that production of these chemokines downstream of IFN γ was not suppressed in NK-cell-depleted mice in our experimental condition. When compared with the experiment on GKO mice, accumulation of CD4-positive cells and CD8-positive cells were more evident in NK-cell-depleted mice than in GKO mice (Supplementary Figure). On the other hand, there was no remarkable difference in the expression of CD31 between pCMV injection and pCMV-IL-12 injection. These results suggested that in NK-cell-depleted mice IL-12 may exert anti-tumor effect via T-cell accumulation rather than anti-angiogenesis.

Since the liver contains an abundance of immune cells (especially NK cells) [31], the cytokine-mediated activation of these cells may be a promising approach toward anti-tumor therapy in this organ [32]. IL-12 is a cytokine

known to elicit a potent anti-tumor effect in mouse experimental models. However, clinical trials attempted to date were interrupted by fatal adverse effects. Systemic IL-12 therapy has been associated with dose-limiting toxicity [33]. IL-12 induces activation of the pro-inflammatory pathway which causes the complications of high dose cytokine, independent of the action of IFN γ [34]. On the other hand, the levels of immunosuppressive cytokine, for example, TGF- β 1 or IL-10 were significantly higher in patients with hepatocellular cancer and colon cancer [35–38]. In particular, TGF- β 1 in serum can limit NK-cell IFN γ production [39]. Thus, in patients with advanced disease, IL-12 may not be able to exert its potent anti-tumor immune-effects because IFN γ , which is an important mediator of the IL-12-induced immune response, is less effective in a tumor environment. In the present study, we demonstrated that NK-cell IFN γ production induced by IL-12 was sufficient for the anti-metastatic effect of IL-12 in the liver. Thus, a strategy of efficiently producing IFN γ from NK cells may be important for avoiding toxicity of IL-12 therapy.

IL-12 gene therapy has an advantage to allow local production of the cytokine at the tumor sites with low serum concentration. Studies demonstrated that intratumoral administration of adenovirus encoding IL-12 to animals with different types of carcinoma caused complete tumor eradication and increased long-term survival [40, 41]. Moreover, injection of IL-12-encoding adenovirus in one nodule of liver tumor resulted in regression of distant nodules in the liver [41]. However, in a clinical trial anti-tumor activity of IL-12-encoding adenovirus was only observed in the injected tumor sites, but not in distant tumors [42]. The present study shed light on hydrodynamic transfection of hepatocytes as a promising strategy to eradicate disseminated tumors from whole liver.

In summary, NK cells are not just an effector for innate immunity but a mediator producing IFN γ that is critical for the IL-12 anti-tumor effects. Extremely higher expression of STAT4 may be a basis for efficient production of IFN γ from NK cells.

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